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16. ABSTRACT

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COMPARISON OF IN VIVO AND IN VITRO METHODS FOR ASSESSING THE EFFECTS OF PHENOBARBITAL ON THE HEPATIC DRUG-METABOLIZING ENZYME SYSTEM

(Model substrate assay; Phase I reactions; Phase II reactions; liver; urinary metabolites; lindane; γ-hexachlorocyclohexane)

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SUMMARY

The effect of daily i.p. injections of 0, 1, 10 and 80 mg/kg phenobarbital for 1 week on the activity of the hepatic drug-metabolizing enzyme system was measured in the rat by a model substrate assay employing lindane (γ-HCH) and by a battery of in vitro enzyme assays. Comparison of the dose-response curves of the in vivo and in vitro assays indicated that urinary metabolites of lindane provided a good index of phenobarbital-induced change in both phase I and phase II reactions.

INTRODUCTION

Studies of unanticipated, persistent and latent side effects from pre- or postnatal exposure to environmental chemicals on the integrity of the hepatic drug-

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Abbreviations: CPMA, 4 chlorophenylmercapturic acid; DCPMA, dichlorophenyl-mercapturic acid; 36/45-HCCH, γ-1,2,3,4,5,6-hexachlorocyclohexene; γ-HCH, γ-hexachlorocyclohexane (lindane); 36/45-PCCH, 2,3,4,5,6-pentachlorocyclohexene; PCCOL, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol; 36/45-TCCH, γ-3,4,5,6-tetrachlorocyclohex-1-ene; TCCOL, tetrachloro-2-cyclohexen-1-ol; TCP, trichlorophenol; TTCP, tetrachlorophenol; TCPMA, trichlorophenylmercapturic acid.

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metabolizing enzyme system are limited. To evaluate these effects, individual animals should be monitored non-invasively as they age. A number of non-invasive techniques are available, including drug-induced sleeping time [1], drug-induced paralysis time [2], ascorbic acid excretion [1], glucaric acid excretion [3–4], 6β-hydroxycortisol excretion [5], expired $^{14}CO_2$ derived from the *N*-demethylation of aminopyrine [6] and drug clearance [7–8]. However, the drug-metabolizing enzyme system involves both phase I reactions, which usually convert an administered chemical to a more polar metabolite and phase II reactions, which conjugate either parent compound or the products from phase I reactions with glucuronate, sulfate, glutathione, glycine, glutamine, or methyl groups. Information derived from the non-invasive assays listed above, however, is limited to an estimation of the magnitude of changes in only the phase I reactions. It would be preferable to analyze changes in the metabolite profile of a chemical biotransformed by both phase I and phase II reactions. This is one of a series of experiments involving inducing agents, inhibitors and hepatotoxins to investigate the applicability of an assay employing γ-hexachlorocyclohexane (lindane) as an in vivo index of toxicant-induced perturbations in xenobiotic metabolism. This study compares the dose response effect of phenobarbital on the excreted profile of lindane metabolites with the responses from a battery of in vitro measurements.

MATERIALS AND METHODS

At both Purdue University, where the in vitro assays were conducted, and at the E.P.A. Environmental Research Lab, where the model substrate assay was employed, individual weanling female Fischer 344 rats (Charles River, Kingston, NY) were housed in stainless steel cages with free access to Purina lab chow and water. A 12:12 h light-dark cycle was maintained and room temperature was kept at 22 ± 2°C. Bed-O-Cobs (Andersons Cob Division, Delphi, IN) was used as bedding in both labs.

After an adaptation period of one week, 4 groups of 6 rats received daily intraperitoneal (i.p.) injections of aqueous solutions containing either 0, 1, 10, or 80 mg/kg sodium phenobarbital (J.J. Baker Co., Phillipsburg, NJ) for 7 days. The dosing volume was 1% of the body weight of the treated animal.

On day 8 of the in vivo study, all rats were dosed subcutaneously (s.c.) with 19.9 mg lindane (containing 3.5 μCi[U-^{14}C]lindane). [U-^{14}C]lindane, with a specific activity of 54 mCi/mmol and a radiochemical purity of 98%, was obtained from Amersham, Arlington Heights, IL. Following treatment on day 8, the animals were transferred to animal containment chambers (Plas-Labs, Lansing, MI) where urine, feces and expired air were collected for 24 h.

A 10-ml urine aliquot was made acid (pH 2.0) with HCl and extracted twice with equal volumes of isooctane for free chlorophenols, alcohols and unaltered lindane. The residual urine was hydrolyzed with β-glucuronidase (Sigma, St. Louis, MO) by

a previously reported method [9] and after acidification (pH 2.0) was extracted twice with equal volumes of isooctane to obtain the chlorophenol and alcohol glucuronides. Residual urine from this extraction was hydrolyzed with arylsulfatase (Sigma) by a previously reported method [9] and, after acidification (pH 2.0), was extracted twice with equal volumes of isooctane to obtain chlorophenol and alcohol sulfates. Residual urine from this extraction was hydrolyzed and acylated as previously described by Allsup and Walsh [10] and extracted twice with isooctane for determination of the mercapturic acid metabolites of lindane. Urine extracts were analyzed for lindane metabolites on a Tracor Model MT-220 gas chromatograph equipped with a ^{63}Ni electron-capture detector and linearizer. The column consisted of a 183 × 0.63 cm O.D. glass U-tube packed with 5% EGS + 1% H_3PO_4 on 90/100-mesh Anakrom A (Analabs, North Haven, CT). The column was maintained isothermally at 170°C. Inlet and detector temperatures were 245°C and 330°C, respectively. The 5% methane/argon carrier gas flow rate was regulated at 55 cm^3/min. Radioactivity was analyzed in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer using a quench correction curve and a scintillation solution containing 500 ml toluene, 500 ml cellosolve, 5.0 g 2,5-di-phenyloxazole (PPO) and 50 mg 1,4-bis-2-(5-phenoxazolyl)-benzene (POPOP).

For the in vitro work, 24 h after the last dose of phenobarbital, blood was obtained from the orbital sinus for measurement of plasma sorbitol dehydrogenase [11]. Whole liver homogenates were used for determination of glucose-6-phosphatase [12] and the glucuronidation of 1-naphthol and chloramphenicol [13]. Microsomal and cytosolic fractions were prepared by differential centrifugation. Ethylmorphine demethylation was measured by the method of LaDu et al. [14], aniline hydroxylation by the method of Imai et al. [15], hexobarbital oxidation by the method of Kupfer and Rosenfeld [16,17] and aldrin epoxidation by the method of Wolff et al. [18]. Glutathione-*S*-transferase activity in the cytosolic fraction was determined using the aryl substrate 1,2-dichloro-4-nitrobenzene and the epoxide substrate 1,2-epoxy-3-(*p*-nitrophenoxy)propane [19]. Proteins were quantified using the method of Lowry et al. [20].

In vivo responses that measured changes in phase I reaction included total excreted alcohols, 2,4,6-trichlorophenol (2,4,6-TCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TTCP), 2,4,5-trichlorophenol (2,4,5-TCP) and 4-chlorophenylmercapturic acid (CPMA) (Fig. 1). Phase I reactions in vitro included aniline hydroxylase, hexobarbital oxidase, ethylmorphine demethylase, and aldrin epoxidase. In vivo indices of altered UDP-glucuronyltransferase activity included excreted chlorophenol glucuronides and excreted alcohol glucuronides (Fig. 1), while assays utilizing chloramphenicol and naphthol as substrates were the in vitro measurements. In vivo changes in sulfotransferase activity were estimated by alterations in the urinary content of chlorophenol and alcohol sulfates (Fig. 1). Urinary mercapturic acid (MA) metabolites (Fig. 1) served as the in vivo indicator of altered glutathione-S-transferase activity while assays employing 1,2-dichloro-4-nitrobenzene and

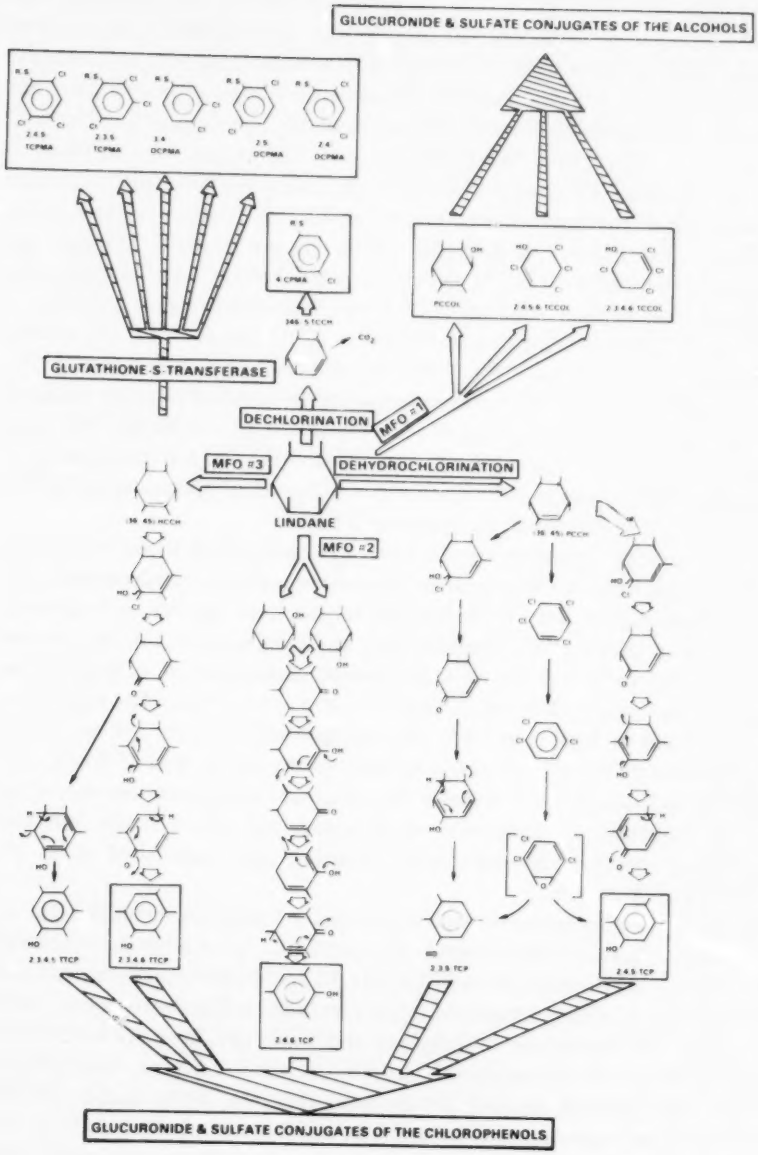

Fig. 1. The biotransformation of lindane in rats. Where the configuration of the metabolites is known, this is indicated by a fractional notation, numbers in the numerator denoting chlorine above and numbers in the denominator denoting chlorine below the plane of the ring.

benzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane were used in vitro.

To compare the dose-response curves from the in vivo and in vitro assays, a least squares best fit of the log of the responses, relative to the control mean, on a logarithmic transform of the dose was determined. Comparable in vivo and in vitro indices were contrasted to determine (1) whether the slope of each response curve was different from zero, (2) whether the slopes of the compared response curves differed from one another, and (3) doses at which the response means differed. Slopes were compared by student's *t*-test [21] while relative response values at each dose were compared by a two-way analysis of variance on the ranks of the data [22] followed by Duncan's Multiple Range Test [23]. Values were considered significantly different at $P \leqslant 0.05$.

RESULTS

The metabolism of lindane by the rat is illustrated in Fig. 1. Cytochrome P-450 catalyzes the biotransformation of lindane to 2,4,6-TCP and 2,3,4,6-TTCP by distinctly different mechanisms [24–26] while oxidation to the alcohols may involve nucleophilic attack of the superoxide anion [27]. Dehydrochlorination of lindane leads to 2,4,5-TCP [27] while dechlorination is the rate-limiting step in the metabolism of lindane to CPMA [28]. It is likely that different isozymes are involved in the conjugation of the chlorophenol and alcohol metabolites. For example, multiple forms of UDP-glucuronyltransferase [29,30] and sulfotransferase [31] are found in the liver and it has been reported that conjugation of *p*-bromophenol, which like the chlorophenols is planar, occurs via a different isozyme of UDP-glucuronyltransferase than does glucuronidation of the monoterpenoid alcohols, which like the lindane-derived alcohol metabolites [29], are bulkier than the phenols.

Tables I and II summarize the comparison of the dose-response relationships from the in vivo and in vitro assays for the phase I and phase II reactions, respectively. Significantly positive slopes were observed for all indices of the phase I reactions except dechlorination, and for all phase II reactions except the sulfate conjugation of the alcohols. Comparisons between the phase I indices at each dose level (Table I) indicated that there were even some in vivo: in vitro responses, such as total excreted alcohols:aniline hydroxylase; excreted 2,4,6-TCP:aniline hydroxylase; and excreted 2,3,4,6-TTCP:hexobarbital oxidase, that were not significantly different at any dose of phenobarbital. These dose-response relationships are illustrated in Fig. 2. On the other hand, the slope and magnitude of aldrin epoxidase were significantly greater than that of the other measurements presented in this table. The effect of phenobarbital pretreatment on dehydrochlorination (excreted 2,4,5-TCP) and dechlorination (excreted CPMA) was not measured in vitro and thus no in vivo:in vitro comparisons were possible. While phenobarbital significantly induced dehydrochlorination of lindane, it had no effect on the dechlorination

TABLE I

EFFECT OF PHENOBARBITAL PRETREATMENT ON PHASE I REACTIONS

Analyses	Phase I reactions							
	Aniline hydroxylation		Ethylmorphine demethylation		Hexobarbital oxidation		Aldrin epoxidation	
	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro
Slope[a]								
Total alcohols	24.7*	17.6*	24.7*	31.5*	24.7*	39.9*	24.7*	124.2*
2,4,6-TCP	26.4*	17.6*	26.4*	31.5*	26.4*	39.9*	26.4*	124.2*
2,3,4,6-TTCP	56.8*	17.6*	56.8*	31.5*	56.8*	39.9*	56.8*	124.2*
2,4,5-TCP	42.4*							
CPMA	13.3							
Comparison of in vivo and in vitro slopes[b]								
Total alcohols								**
2,4,6-TCP								**
2,3,4,6-TTCP		**		**				**
Doses at which responses differ[c]								
Total alcohols				80		1,80		10,80
2,4,6-TCP				80		80		1,10,80
2,3,4,6-TTCP		80		10				1,10

[a] The slope of the dose-response relationship was determined by a least-squares best fit of the log of the response relative to the control mean on a logarithmic transform of the dose. Slopes significantly ($P<0.05$) different from zero by t-test are indicated by*. There were no comparable in vitro responses for dehydrochlorination (excretion of 2,4,5-TCP) or dechlorination (excretion of CPMA).

[b] Slopes were compared by a t-test and significant ($P<0.05$) differences are indicated by **.

[c] Response means for each pathway were compared using analysis of variance on the ranks of the responses followed by Duncan's Multiple Range Test ($P<0.05$)

of the pesticide. As an index of altered UDP-glucuronyltransferase activity, the excretion of the chlorophenol glucuronides more closely resembled the in vitro assay employing the substrate chloroamphenicol while the excretion of the alcohol glucuronides was closer to that of the in vitro assay that used naphthol (Table II). These dose-response curves are depicted in the upper half of Fig. 3. The dose-response effect of phenobarbital on the excretion of mercapturic acid metabolites agreed in slope with the in vitro asays for glutathione-*S*-transferase, but differed from them in response magnitude at some doses. The in vivo data appeared to be closer to that of the in vitro assay involving 1,2-dichloro-4-nitrobenzene as the substrate. These curves are illustrated in the lower half of Fig. 3.

Since sulfotransferase activity was not measured in vitro, no in vivo:in vitro comparisons were possible for the excreted sulfate conjugates. However, the in vivo data indicated that phenobarbital selectively induced the conjugation of the chlorophenols, but not the alcohols.

TABLE II

EFFECT OF PHENOBARBITAL PRETREATMENT ON PHASE II REACTIONS

Analyses	Phase II reactions							
	UDP-glucuronyltransferase				Glutathione-*S*-transferase			
	Chloramphenicol		Napthol		1,2-dichloro-4-nitrobenzene		1,2-epoxy-3-(*p*-nitrophenoxy) propane	
	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro	In vivo	In vitro
Slope[a]								
Chlorophenol glucuronides	53.7*	34.5*	53.7*	12.9*		—		—
Alcohol glucuronides	23.6*	34.5*	23.6*	12.9*		—		—
Total mercapturic acids		—		—	31.8*	23.4*	31.8*	10.7*
Chlorophenol sulfates	34.9*							
Alcohol sulfates	– 15.9							
Comparison of in vivo and in vitro slopes[b]								
Chlorophenol glucuronides				**		—		—
Alcohol glucuronides						—		—
Total mercapturic acids		—		—				
Doses at which responses differ[c]								
Chlorophenol glucuronides				10,80		—		—
Alcohol glucuronides		80		10		—		—
Total mercapturic acids		—		—		10		10,80

[a] The slope of the dose-response relationship was determined by a least-squares best fit of the log of the response relative to the control mean on a logarithmic transform of the dose. Slopes significantly ($P<0.05$) different from zero by *t*-test are indicated by *. There were no comparable in vitro responses for the sulfation of alcohol and chlorophenol metabolites.

[b] Slopes were compared by a t-test and significant ($P< 0.05$) differences are indicated by **.

[c] Response means for each pathway were compared using analysis of variance on the ranks of the responses followed by Duncan's Multiple Range Test ($P<0.05$).

DISCUSSION

The dose response effect of 0, 1, 10 and 80 mg/kg phenobarbital on the drug-metabolizing enzyme system was determined by a non-invasive model substrate assay and by a battery of in vitro enzyme assays. The dose-response relationships for both the in vivo and in vitro assays exhibited a positive trend with dose. There were few significant differences between the slopes of comparable in vivo and in vitro responses and when standardized relative to control means, the magnitude of some in vivo:in vitro responses was not significantly different at any dose of phenobarbital (Tables I and II). Thus, select urinary lindane metabolites provide

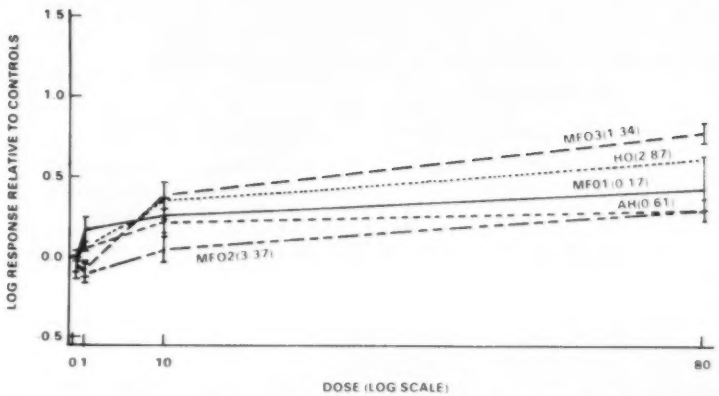

Fig. 2. Comparison of in vivo dose-response curves MFO_1 (excreted alcohols), MFO_2 (excreted 2,4,6-TCP) and MFO_3 (excreted 2,3,4,6-TTCP) with in vitro dose-response curves for aniline hydroxylase (AH) and hexobarbital oxidase (HO). For the in vivo responses, the numbers in parentheses represent the mean % of administered lindane excreted as metabolite/24 h by the control rats. For the in vitro responses, the numbers in parentheses represent the nmoles of substrate oxidized/mg protein/min by liver prepartions from control rats. Each point represents the mean of 6 animals. The vertical lines indicate SE.

satisfactory indices of phenobarbital-induced changes in both phase I and phase II reactions.

It is considered expedient to measure mixed function oxidase reactions with substrates such as hexobarbital, which give type I binding spectra (peak at 380–390 mm and trough at 400 nm) as well as substrates such as aniline, which give type II binding spectra (peak at 440 nm and trough at 400 nm) [32]. Thus it was fortuitous that phenobarbital-induced increases in aniline hydroxylase paralleled an elevation in the urinary content of lindane derived alcohol metabolites and 2,4,6-TCP (Table I, Fig. 2) while increases in the excretion of 2,3,4,6-TTCP mimicked the induction of hexobarbital oxidase activity (Table I, Fig. 2).

Since the planar chlorophenols and the bulkier alcohol metabolites may be conjugated by different isozymes of UDP-glucuronyltransferase and sulfotransferase [29], the assay has the potential to distinguish between induction and inhibition of different forms of these enzymes.

There were differences in magnitude between the in vivo and in vitro responses at certain doses for the estimation of UDP-glucuronyltransferase and glutathione-*S*-transferase (Table II, Fig. 3). In general, the magnitude of the in vivo response was higher although the glucuronidation of the alcohol metabolites was intermediate between the responses of the in vitro assays using chloramphenicol and naphthol (Fig. 3). Nevertheless, based on the slope of the dose-response curves, the estimation of changes in the activity of these phase II reactions by the model substrate assay was not significantly different from that obtained by using the appropriate in vitro enzyme assay.

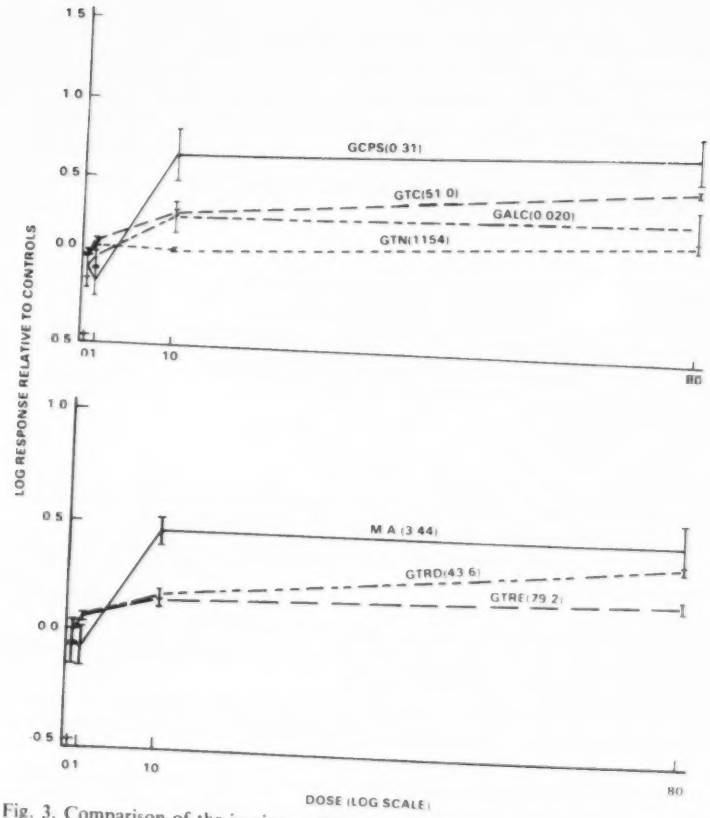

Fig. 3. Comparison of the in vivo and in vitro dose-response curves for the Phase II reactions. (Top) In vivo responses GCPS (excreted chlorophenol glucuronides), and GALC (excreted alcohol glucuronides) were compared with assays for UDP-glucuronytransferase employing chloramphenicol (GTC) and naphthol (GTN) as substrate. (Bottom) In vivo excretion of mercapturic acid (MA) was compared with assays for gluathione-*S*-transferase using 1,2-dichloro-4-nitrobenzene (GTRD) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (GTRE) as substrates. For the in vivo responses, the numbers in parentheses represent the mean % of administered lindane excreted as conjugate/24 h by control rats. For the in vitro responses, the numbers in parentheses represent the mean nmol of conjugated substrate produced per min by liver preparations from control rats. Each point is the mean of 6 animals. Vertical lines indicate SE.

Results from this study indicate that metabolism of the model substrate lindane provides a satisfactory index to phenobarbital-induced changes in both phase I and phase II reactions in the rat. Potential advantages of this assay include (1) noninvasive measurements, (2) the feasibility of monitoring individual animals as they age, (3) economical estimation of alterations in multiple phase I and phase II reactions in the same animal within the same experiment, (4) the possibility of simultaneously monitoring changes in the activity of different forms of the same en-

zyme, (5) enhanced potential for detecting toxicant-induced lesions in the drug-metabolizing enzyme system due to the large number of metabolic pathways examined, (6) avoidance of erroneous estimates due to disruption of phospholipid membranes, loss of permeability barriers, loss of local charge effects, lack of normal clearance mechanisms, and the necessity of distinguishing between changes in metabolism and changes in receptor response, and (7) relatively simple extraction and analytical procedures.

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